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Microbiological and epidemiological studies of *Enterococcus faecium* resistant to amoxycillin in a university hospital in eastern France

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ABSTRACT

Over 3 years, during six 1-month periods of surveillance, 69 cases of *Enterococcus faecium* colonisation or infection were detected in a university hospital in eastern France. Thirty-two cases involved strains resistant to amoxycillin (crude incidence of 0.21/1000 patient-days). The risk of infection with *E. faecium* was higher if the patient was hospitalised in a haematology ward and/or treated with cephalosporins. Amoxycillin-resistant isolates (AmRE) were isolated from different wards and time periods, and none of the characteristics studied were associated significantly with amoxycillin resistance. Amoxycillin-sensitive and -resistant isolates were characterised by pulsed-field gel electrophoresis. Three epidemic patterns were identified which contained 87.5% (28/32) of the AmRE isolates, indicating that clonal spread was responsible, at least partially, for the high incidence of AmRE in this hospital.

Keywords *Enterococcus faecium*, resistance to amoxycillin, molecular epidemiology, risk factors

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INTRODUCTION

Enterococci are now established firmly as major nosocomial pathogens. Bacteria of the genus *Enterococcus* are the fourth most common cause of hospital-acquired infection and the third most common cause of bacteraemia in the USA [1]. The treatment of choice for these infections is usually a synergic combination of a penicillin with an aminoglycoside or a glycopeptide. With the emergence of strains displaying resistance to glycopeptides, mostly *Enterococcus faecium* (VRE), multiple resistance to all currently approved antimicrobial agents is now possible in enterococcal strains [2–4]. Such strains with multiple resistance have caused major outbreaks within hospitals in the USA, and have also transferred from one hospital to another [5–7].

In France, vancomycin resistance in *E. faecium* isolates does not seem to be a major problem at the moment [8–10]. In contrast, a large but variable proportion of *E. faecium* isolates has acquired high-level resistance to aminoglycosides and resistance to amoxycillin [8–10]. The mean reported rates of resistance to amoxycillin for *E. faecium* hospital isolates in various countries range from 23.3% to 98.7% [8–11]. Resistance affects the current treatment of enterococcal infections. Moreover, in the USA, amoxycillin resistance in *E. faecium* has been proposed as a risk factor for the spread of vancomycin resistance, with the genetic element conferring amoxycillin resistance being sometimes linked to vancomycin resistance transposons, such as Tn*5382* [12].

In hospitals, the emergence and spread of resistant pathogens can be limited by improving management procedures; e.g., by isolating carriers or infected patients to prevent cross-colonisation, and by implementing antibiotic policies to reduce the selection of resistant bacteria during treatment [13]. The efficacy of such strategies to control resistance depends on the epidemiology of the resistant bacteria in a given institution [14].

This study reports the rates of infection with *E. faecium* and the level of resistance among isolates from Besançon University Hospital in eastern France. The epidemiological and microbiological characteristics of the amoxycillin-resistant isolates (AmRE) obtained during the

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six 1-month periods of the survey were determined.

MATERIALS AND METHODS

Setting

Besançon Hospital is a public university hospital with 1320 beds in acute care facilities comprising three intensive care units (ICUs), 16 general medical and 11 surgical wards. Around 50 000 patients are admitted each year, giving 355 000 patient-days of hospitalisation/year.

Study design

Clinical cultures (excluding stool cultures) from all patients admitted to the hospital were examined to identify possible cases of *Enterococcus* colonisation or infection, in a non-sequential study (six 1-month periods: November 1995 = A; May 1996 = B; November 1996 = C; May 1997 = D; November 1997 = E; May 1998 = F). *Enterococcus* isolates were identified to species level (see below). Amoxycillin-susceptible and amoxycillin-resistant *E. faecium* isolates were characterised by pulsed-field gel electrophoresis (PFGE). Patients with positive cultures were compared in two case-control studies to identify risk factors for colonisation or infection with *E. faecium* strains, and for colonisation or infection with amoxycillin-resistant *E. faecium* strains.

Bacterial strains

Enterococci were identified to the species level, using the API 20 Strep system (bioMérieux, Lyon, France). Standardised disk diffusion tests were performed to determine susceptibility to erythromycin, chloramphenicol, co-trimoxazole, tetracycline, and amoxycillin according to the criteria recommended by the Antibiogram Committee of the French Society for Microbiology (MIC breakpoints of 4 and 16 mg/L) and the NCCLS [15,16]. Isolates were tested for β-lactamase production with nitrocefin. High-level aminoglycoside resistance was detected by breakpoint screening on Mueller-Hinton agar (MHA; BBL, Cockeysville, MD) containing kanamycin (1000 mg/L) (HLKR) or gentamicin (500 mg/L) (HLGR). Such high-level resistance in clinical enterococcal isolates is usually mediated by various aminoglycoside-modifying enzymes causing resistance to amikacin if kanamycin resistance is detected, and to most commercially available aminoglycosides if gentamicin resistance is detected. The MICs of penicillin, amoxycillin ± clavulanic acid, ampicillin, piperacillin ± tazobactam, imipenem, vancomycin and teicoplanin were determined by the Etest method (BMD, Marne-la-Vallée, France).

PCR

Glycopeptide-resistant enterococci were tested for the presence of the *van*A and *van*B resistance genes by PCR. DNA was extracted by the boiling method of Elaichouni *et al*. [17]. PCR assays were performed as described by Dutka-Malen *et al*. [18]. Two different primer pairs (*van*A, *van*B) [18] were used in the assay (50 pmol of each individual primer/reaction, with initial denaturation at 92 °C for 2 min, followed by 30 cycles of 1 min at 92 °C, 1 min at 54 °C and 1 min at 72 °C. Amplicons were analysed by electrophoresis on agarose 1% w/v gels with a DNA ladder (Life Technologies, Gaithersburg, MD, USA) used as a size standard. *E. faecium* BM4147 (*van*A) and *E. faecium* V583 (*van*B) were used as reference strains. Gels were stained with ethidium bromide.

Genotyping

Genomic DNA digested with *Sma*I was subjected to PFGE, as described by Murray *et al*. [19,20] using a clamped homogeneous electric-field apparatus (CHEF-DRIII; Bio-Rad, Hercules, CA, USA). Samples of *Sma*I-restricted *Staphylococcus aureus* NCTC 8325 DNA were included in each run as an internal standard. Electrophoretic restriction patterns were analysed by scanning photographic negatives. GelCompar software was used for cluster analysis (Applied Maths Kortrijk, Belgium) with the Dice correlation coefficient, and a dendrogram was produced using the UPGMA (unweighted pair group method using arithmetic averages) clustering algorithm. Major restriction patterns were defined as patterns differing by more than six fragments [21], and were each designated with a number, with each variant indicated by a suffix letter. Epidemic patterns were defined as patterns corresponding to isolates from more than two patients. Unique patterns were defined as patterns corresponding to only one isolate.

Epidemiological data and definitions

Data were recorded concerning the ward (type of ward and number of patients admitted) and the patient (age, sex, previous hospital admissions, duration of hospital stay and antibiotic treatment in the 7 days preceding strain isolation). All the enterococci included in the study were from clinical specimens, but isolates from stool samples were excluded. No clinical information was collected to differentiate colonisation from true infections, as the objective of the study was centred on the frequency of resistance to amoxycillin and not on the virulence of strains.

The main outcome was the incidence of *Enterococcus* colonisation or infection. Crude incidence was estimated by dividing the total number of cases of *Enterococcus* colonisation or infection by the total number of exposed patients. The time required for colonisation or infection by *E. faecalis* and *E. faecium* strains, and for AmSE and AmRE strains was compared.

Risk factors

Two multivariate cohort analyses were performed to identify risk factors for colonisation or infection with *E. faecium* (the control cohort contained patients colonised or infected with *Entercoccus faecalis*), and for colonisation or infection with amoxycillin-resistant *E. faecium* strains (with the control cohort being patients colonised or infected with *E. faecium* strains susceptible to amoxycillin). The potential risk factors studied were: sex, age, origin of the patient (transfer from another hospital), ward in which the patient was hospitalised, duration of hospitalisation before colonisation or infection, and antibiotic therapy before colonisation or infection. Previous antibiotic treatment was first analysed as a factor in itself and was then analysed according to the agent used.

Odds ratios were estimated from regression coefficients and 95% confidence intervals (CIs) were calculated. The χ^2 test was used to test correlations between variables, with $p < 0.05$ significant. To adjust for confounding factors, variables with p values between 0.05 and 0.1 in univariate analyses were added to multiple regression models. Statistical analysis was performed using the Epi-Info and BMDP software packages.

RESULTS

Incidence

During the study, 24 228 patients were admitted to the hospital for a total of 150 111 days. In total, 799 cases of *Enterococcus* colonisation or infection were observed in 737 patients, giving crude incidences of colonised or infected patients and of colonisation or infection of 3.04% and 3.30%, respectively, and a crude incidence of colonisation or infection of 5.32/1000 days of hospitalisation. Of these 799 cases of colonisation or infection, 69 involved *E. faecium* strains in 67 patients. All 32 strains showing resistance to amoxycillin were *E. faecium* strains, accounting for 46.4% of the *E. faecium* strains. This gave crude incidences of 0.46/1000 patient-days for all *E. faecium* strains (from 0.43 to 0.51 according to the period) and 0.21/1000 patient-days for AmRE (from 0.19 to 0.24 according to the period). Table 1 shows the distribution of cases according to ward and to sites of colonisation or infection. The incidence of *E. faecium* colonisation or infection differed considerably between wards ($p < 10^{-5}$), whereas no difference was observed in the frequency of amoxycillin resistance.

Table 1. Distribution of cases of colonisation or infection with *Enterococcus faecium*

	Colonisation or infection with *E. faecium*		Colonisation or infection with *E. faecium* strains showing resistance to amoxycillin	
	Number (frequency[a])	Incidence[b]	Number (frequency[c])	Incidence[b]
Wards				
Haematology	15 (17.9)	2.54	7 (46.7)	1.18
Urology	7 (6.9)	1.35	4 (57.1)	0.77
Intensive care	9 (12.0)	2.00	5 (55.5)	1.11
Paediatric	4 (7.7)	0.80	3 (75.0)	0.60
Other medical wards ($N = 14$)	20 (7.5)	0.29	8 (40.0)	0.11
Other surgical wards ($N = 10$)	14 (6.4)	0.23	5 (35.7)	0.08
Sites of isolation				
Urinary tract	47 (8.7)	0.31	23 (48.9)	0.15
Pus	3 (6.8)	0.02	2 (66.7)	0.013
Bloodstream	1 (8.3)	0.007	0 (0.0)	0.00
Bronchopulmonary tract	1 (7.7)	0.007	0 (0.0)	0.00
Other	17 (9.1)	0.11	7 (41.2)	0.05

[a]Frequency in the *Enterococcus* genus (%); [b]Incidence/1000 patient-days; [c]Frequency in *E. faecium* (%).

Resistance

None of the AmRE strains produced a β-lactamase. The MICs of various β-lactam antibiotics are shown in Table 2. Table 3 shows the frequency of co-resistance in AmRE and AmSE isolates, and compares these values to the frequency of resistance in *E. faecalis* isolates. Only one *E. faecium* isolate was resistant to vancomycin (MIC > 256 mg/L) and teicoplanin (MIC > 256 mg/L) and was identified as the *van*A type by PCR. This strain was co-resistant to amoxycillin, kanamycin, erythromycin and co-trimoxazole.

Molecular epidemiology

The 69 *E. faecium* isolates typed yielded 37 major DNA patterns: 29 unique patterns (each associated with one patient), five patterns corresponding to two isolates, and three epidemic patterns. Thirty major DNA patterns were identified among the 37 amoxycillin-susceptible iso-

Table 2. MICs (mg/L) of various β-lactam antibiotics for AmSE and AmSE isolates

	β-lactam tested						
E. faecium isolates	Penicillin	Ampicillin	Amoxycillin	Amoxycillin/ clavulanate	Piperacillin	Piperacillin/ Tazobactam	Imipenem
AmSE ($n = 37$)							
Mean MIC	8.07	1.06	0.74	0.73	17.18	13.88	7.91
SD	18.62	1.33	0.78	0.87	29.91	21.96	12.73
MIC_{50}	2	0.5	0.5	0.38	6	6	0.75
MIC_{90}	16	2	1.5	1.5	32	32	> 32
Range	0.016–96	0.032–6	0.032–3	0.032–4	0.38–128	0.38–96	0.047 – > 32
AmRE ($n = 32$)							
Mean MIC	247.80	59.90	26.15	29.70	> 256	> 256	> 32
SD	37.71	65.73	22.15	41.54	0	0	0
MIC_{50}	> 256	32	16	16	> 256	> 256	> 32
MIC_{90}	> 256	128	64	48	> 256	> 256	> 32
Range	48 – > 256	16 – > 256	8–96	6 – > 96	> 256	> 256	> 32

AmSE, amoxycillin-susceptible *Enterococcus*; AmRE, amoxycillin-resistant *Enterococcus;* SD, standard deviation.

Table 3. Frequency of co-resistance amongst enterococci

	E. faecium isolates				Total *Enterococcus* isolates			
	AmSE (n = 37) n (%)	AmRE (n = 32) n (%)	RR[a]	p	*E. faecalis* (n = 669) n (%)	*E. faecium* (n = 69) n (%)	RR[a]	p
Gentamicin[b]	5 (13.5)	5 (15.6)	1.16	NS	49 (7.0)	10 (14.5)	2.07	0.025
Kanamycin[b]	17 (45.9)	27 (84.4)	1.84	0.0009	382 (54.6)	44 (63.8)	1.17	NS
Erythromycin	32 (86.5)	32 (100)	1.16	NS	566 (81.0)	64 (92.7)	1.15	0.015
Chloramphenicol	14 (37.8)	19 (59.4)	1.57	NS	401 (57.4)	33 (47.8)	0.83	NS
Tetracycline	20 (54.1)	30 (93.7)	1.73	0.0002	521 (74.5)	50 (72.5)	0.97	NS

[a]RR, relative risk; [b]High-level.

lates, and seven among the 32 AmRE isolates. Twenty-eight AmRE isolates and two AmSE had epidemic patterns, and three AmRE isolates and 26 AmSE had unique patterns (one AmRE and nine AmSE isolates had patterns corresponding to two isolates) (relative risk: 16.19; 95% CI: 4.18–62.71). Some DNA patterns were shared by isolates with different antibiotic susceptibility phenotypes. Three major epidemic patterns comprised 21 (19 AmRE and two AmSE), six AmRE and three AmRE isolates, respectively. AmRE isolates were from different wards and different periods of the study and the respective colonisation or infection events occurred during and after the first 48 h following admission (Fig. 1). The *van*A isolate belonged to an epidemic pattern comprising 21 isolates with six different antibiotic susceptibility phenotypes (susceptible, AmSE-HLGR-HLKR-vancomycin^S, AmRE-Low-level GR (LLGR)-Low-level KR (LLKR)-vancomycin^S, AmRE-HLGR-HLKR-vancomycin^S, AmRE-LLGR-HLKR-vancomycin^S, AmRE-LLGR-HLKR-vancomycin^R). The frequency of isolates yielding epidemic patterns did not differ significantly between haematology wards (56.3%) and other wards (39.6%) (relative risk: 1.67; 95% CI: 0.70–3.97).

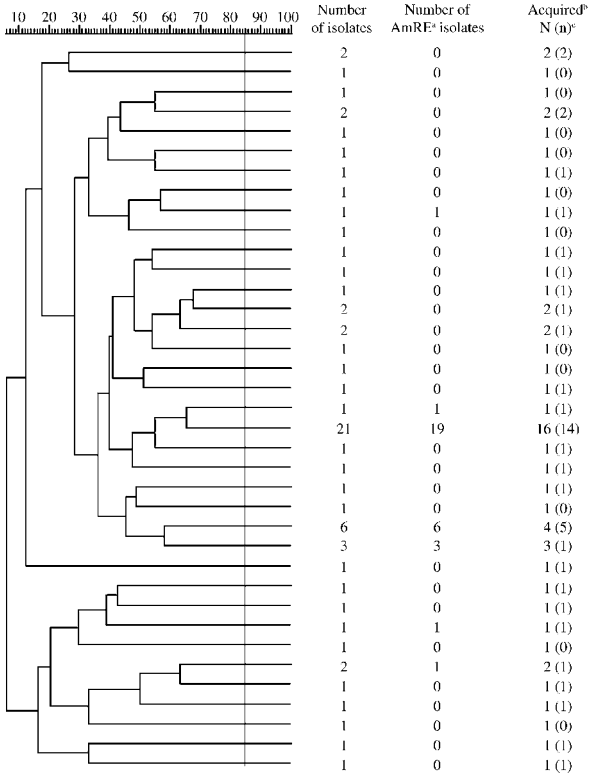

Fig. 1. DNA fragment pattern similarity and epidemiological information for *Enterococcus faecium* isolates (patterns differing by more than six fragments). [a]AmRE, amoxycillin-resistant *Enterococcus*; [b]Colonisation or infection acquired after the first 48 h of hospitalisation; [c]N, number of hospital-acquired (nosocomial); n, number of hospital units or wards.

Clinical epidemiology

Clinical records were available for the 67 patients colonised or infected with *E. faecium* and for 567 of the 737 patients colonised or infected with *E. faecalis* (Table 4). The variables associated significantly with *E. faecium* colonisation or infection in both univariate and multivariate analyses are listed in Table 5. The risk of colonisation or infection with *E. faecium* was higher if the patient was hospitalised in a haematology ward or treated with cephalosporins. None of the characteristics studied were associated significantly with infection with AmRE isolates after univariate analysis; therefore multivariate analysis was not performed.

DISCUSSION

The frequency of amoxycillin resistance amongst enterococci in our institution was very high, as has also been reported in other countries (20%–90%) [9–11]. The study design, based on 1-month periods of surveillance every 6 months, facilitated

Table 4. Main characteristics of patients included in the study

	Colonisation or infection with			
	E. faecalis (*n* = 567) n (%)	*E.faecium* (*n* = 67) n (%)	AmSE[a] (*n* = 36) n (%)	AmRE[a] (*n* = 31) n (%)
On admission				
Age, yrs[b]	52 (25)	49 (26)	53 (25)	46 (28)
Men	282 (49.7)	29 (43.3)	17 (47.2)	12 (38.7)
Transfer from another hospital	61 (10.8)	3 (4.5)	1 (2.8)	2 (6.5)
During hospitalisation				
Colonisation or infection				
Acquired[c]	351 (61.9)	45 (67.2)	23 (63.9)	22 (71.0)
Delay to acquisition (days)[b]	9.9 (14.8)	14.1 (18.9)	15.8 (23.4)	12.0 (12.0)
Ward				
Haematology	49 (8.6)	14 (20.9)	8 (22.2)	6 (19.4)
Urology	80 (14.1)	7 (10.4)	3 (8.3)	4 (12.9)
Intensive care	56 (9.9)	9 (13.4)	4 (11.1)	5 (16.1)
Paediatric	34 (6.0)	4 (6.0)	1 (2.8)	3 (9.7)
Other medical wards	185 (32.7)	21 (31.3)	12 (33.3)	9 (29.0)
Other surgical wards	163 (28.7)	12 (17.9)	8 (22.2)	4 (12.9)
Antimicrobial therapy				
No antibiotic	444 (78.3)	39 (58.2)	21 (58.3)	18 (58.1)
Penicillin	46 (8.1)	5 (7.5)	3 (8.3)	2 (6.5)
Cephalosporin	43 (7.6)	20 (29.9)	10 (27.8)	10 (32.5)
Fluoroquinolone	26 (4.6)	4 (6.0)	4 (11.1)	0 (0)
Aminoglycoside	28 (4.9)	8 (11.9)	3 (8.3)	5 (16.1)
Metronidazole	19 (3.4)	7 (10.4)	6 (19.4)	1 (3.2)

[a]AmSE, amoxycillin-susceptible *Enterococcus faecium*; AmRE, amoxycillin-resistant *Enterococcus faecium*.
[b]For continuous variables, mean values (± standard errors) are given.
[c]Colonisation or infection acquired after the first 48 h of hospitalisation.

Table 5. Univariate and multivariate analyses of factors associated with *Enterococcus faecium* colonisation or infection

	Univariate analysis		Multivariate analysis	
Risk factor	**Odds Ratio**	**CI 95%[a]**	**Odds Ratio**	**CI 95%[a]**
Prior administration of antibiotics	2.56	1.52–4.32	NS	–
Cephalosporins	5.11	2.79–9.33	5.05	2.74–9.31
Aminoglycosides	2.66	1.16–6.08	NS	–
Metronidazole	3.21	1.31–7.85	NS	–
Hospitalisation in a haematology ward	2.61	1.36–5.00	2.55	1.30–5.02

[a]CI 95%, 95% confidence interval.

the collection and the analysis of data and strains in real time, but may misdiagnose outbreaks and either overestimate or underestimate the frequency of resistance. In the present study, no significant differences were observed over the 3-year study period, thereby, establishing the validity of the high frequency of resistance observed. Moreover, this frequency of amoxycillin resistance among *E. faecium* isolates did not differ between wards (Table 1) or according to the time of acquisition (Table 4). Thus, it is not necessary to take into account the time of acquisition, or the type of ward, when deciding how to treat infections. Analysis of strains expressing very high levels of amoxycillin resistance, as in this study, suggests that the increase in resistance is caused by mutations decreasing the binding affinity of PBP5 for amoxycillin. This also results in high-level resistance to other major β-lactam antibiotics, such as piperacillin, amoxycillin ± clavulanate and imipenem (Table 2) [22]. Such high-level resistance to β-lactams may pose problems in terms of treatment, especially if, as frequently observed (Table 3), it is associated with high-level resistance to aminoglycosides.

In this study, the frequency of resistance to imipenem and to piperacillin was higher than that of resistance to amoxycillin; i.e., amoxycillin-susceptible strains exhibited resistance to piperacillin and imipenem. Rybkine *et al*. [22] demonstrated that MICs of ampicillin were lower than those of piperacillin and imipenem; similarly, El Amin *et al*. in a Swedish hospital [23] and Brandt *et al*. in Switzerland [24] described resistance to imipenem in ampicillin-susceptible strains. The observed difference between MICs of amoxycillin and MICs of amoxycillin-clavulanate was probably explained by the higher saturation of PBP5 by the combination [22].

The high prevalence of AmRE isolates in our hospital was caused partly by clonal spread of *E. faecium* strains, since >85% of AmRE isolates shared three PFGE patterns (Fig. 1). Isolates acquired after the first 48 h of hospitalisation were observed with identical patterns throughout the study, suggesting long-term transmission within the hospital, as demonstrated in the study of Fortún *et al*. [25]. However, there was also evidence for the clonal dissemination of some epidemic strains out of our hospital since the prevalence of AmRE strains was no lower among isolates responsible for colonisations or infections acquired during the first 48 h than among colonisations or infections acquired after 48 h of hospitalisation. However, the classification of cases does not exclude the possibility that patients could have acquired AmRE during a previous hospital stay.

An association between the use of antibiotics, such as extended-spectrum cephalosporins, and infection with enterococci, particularly AmRE, has been reported in different studies [25–30]. The methodology used in some studies [26,27] may have led to the identification of cephalosporins as a risk factor for AmRE by chance because the cases (patients infected with AmRE) were compared to patients without *Enterococcus* infection. Therefore,

two risk factor analyses were conducted, first to identify significant factors for colonisation or infection with *E. faecium*, and second to identify significant factors for colonisation or infection with AmRE. In our study, two previously identified risk factors for *E. faecium* bacteraemia [31] were linked significantly with colonisation or infection with *E. faecium*: namely previous administration of cephalosporins (relative risk of 5) and hospitalisation in a haematology unit (relative risk of 2.5). Fortún *et al*. [25] identified previous administration of β-lactams as a risk factor for bacteraemia with AmRE, comparing patients infected with AmRE to patients infected with AmSE. In contrast, no risk factor was identified as significantly associated with colonisation or infection with AmRE in the present study. First, gastrointestinal carriage of enterococci is frequent and previous carriage of the resistant strain cannot be excluded [26]. Second, the AmRE isolates were co-resistant to non-β-lactam antibiotics (Table 3). So, all antibiotic treatment, other than broad-spectrum penicillins, reduces the power of the analysis. However, it is clear that the selective pressure exerted by all antibiotics except glycopeptides favoured the isolation of these strains. Moreover, AmRE clones have a considerable potential to disseminate, as demonstrated by the high frequency of isolates yielding epidemic patterns.

Vancomycin resistance does not seem to be a major problem in our hospital at present, although multiresistant enterococci are endemic. Total vancomycin consumption is lower in our hospital than in American hospitals, but vancomycin consumption is high in haematology wards, in which the incidence of AmRE colonisation or infection is also high [32]. In these wards, once the *van*A gene appears on a plasmid, its dissemination is encouraged by increasing use of glycopeptides and of other antibiotics. It would therefore be of value to perform a prospective survey of clinical isolates of *E. faecium* in our hospital.

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